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Citation for final published version:

Orr, Carl, Sousa, Elsa, Boyle, David L., Buch, Maya H., Buckley, Christopher D., Cañete, Juan D., Catrina, Anca I., Choy, Ernest ORCID: <https://orcid.org/0000-0003-4459-8609>, Emery, Paul, Fearon, Ursula, Filer, Andrew, Gerlag, Danielle, Humby, Frances, Isaacs, John D., Just, Søren A., Lauwerys, Bernard R., Le Goff, Benoit, Manzo, Antonio, McGarry, Trudy, McInnes, Iain B., Najm, Aurélie, Pitzalis, Constantino, Pratt, Arthur, Smith, Malcolm, Tak, Paul P., Thurlings, Rogier, Fonseca, João E. and Veale, Douglas J. 2017. Synovial tissue research: a state-of-the-art review. *Nature Reviews Rheumatology* 13 (8) , pp. 463-475. 10.1038/nrrheum.2017.115 file

Publishers page: <http://dx.doi.org/10.1038/nrrheum.2017.115>  
<<http://dx.doi.org/10.1038/nrrheum.2017.115>>

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## Synovial Tissue Research: State of the Art Review

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## **Abstract**

The synovium is the major target tissue of inflammatory arthritides such as rheumatoid arthritis (RA). The study of synovial tissue has advanced significantly over a number of decades from arthroplasty, blind needle biopsy and more recently facilitated by arthroscopic and ultrasonographic technology that allows easier visualisation and improves the reliability of obtaining synovial biopsies. The potential for study of pathogenesis, patient stratification, discovery of biomarkers and novel targets, as well as validation of therapy, have all been progressed rapidly in the last decade, facilitated by increasingly diverse and sophisticated analytical and technological approaches. In this review we describe clinical and translational developments in the field of synovial tissue research, outlining current and novel investigative technologies, and highlight their application to advance our understanding of the foregoing imperatives.

# Introduction

## 1.1 General Considerations

Chronic inflammatory arthritides (IA) comprise a heterogeneous group of diseases characterised by inflammation of the synovium, often accompanied by destruction of adjacent cartilage and bone. Inflammation is characterised by synovial neovascularisation, stromal proliferation and leukocyte extravasation.<sup>1</sup> For the purpose of this review we will focus on rheumatoid arthritis (RA), due to its prevalence, and there being the most extensive body of research on this common cause of synovitis. RA is usually persistent and progressive, leading to joint damage, disability and deformity if left untreated. RA is associated with a reduction in quality of life as well as decreased longevity and constitutes an important burden on healthcare spending.<sup>2-4</sup>

Recent years have seen several dramatic advances in the treatment of IA in general, and particularly in RA. The patterns of clinical response are remarkably similar for agents with different targets challenging our understanding of disease mechanisms. While it is true that the last two decades have witnessed an unprecedented, if qualified, success in treating RA, a substantial portion of patients still do not achieve low disease activity or remission.<sup>5,6</sup>

The main challenges in biomedicine and translational research in RA focus on early diagnosis, personalised medicine, and the development of meaningful outcome assessments.<sup>7</sup> It is logical to hypothesise that each of these aims can be facilitated by the identification and development of appropriate biomarkers. However, while peripheral blood biomarkers such as rheumatoid factor and anti-citrullinated peptide antibodies (ACPA) have been shown to be relatively specific and may predict development of RA in asymptomatic individuals,<sup>8,9</sup> they may only be found in 70-80% of RA subjects. Indeed, beyond these factors our repertoire of blood biomarkers to assist with diagnosis, disease progression and response to therapy is currently extremely limited.<sup>10,11</sup>

Since the synovium is the principal target of inflammation in RA, and the specific resident fibroblast-like synoviocyte (FLS) is implicated in the pathogenesis of synovitis, one promising approach in the search for biomarkers may be the detailed analysis of the inflamed synovial tissue. Using a combination of established methodologies, together with incorporating new high-throughput technologies with the capability of detailed examination of genes and their products on a scale never before possible, a new opportunity awaits in the search for these biomarkers.

## 1.2 Anatomy and Physiological Regulation of the Synovial Joint

The synovial joint comprises opposing bones, with the articular surface covered by cartilage. The main protein in bone is type I collagen, while cartilage, which is found on the articulating surfaces, comprises mainly type II collagen and proteoglycan molecules. The non-articulating surfaces are lined by a thin adventitious layer known as synovium. Normal synovial tissue comprises 1 to 3 layers of specialized columnar FLS interspersed with macrophages.<sup>12</sup> The entire structure is housed by a fibrous capsule, and together with ligaments, muscles and tendons, this confers strength and stability to the joint.

Several factors contribute to the maintenance of normal homeostasis in the synovial joint. These include normal expression of the protective lubricin<sup>13</sup>, FLS secretion of matrix metalloproteinases (MMPs), immune sentinel roles played by resident macrophages and FLS, the regulated entry and exit of leukocytes involved in immune surveillance, and local regulation by cytokines and growth factors.

Cytokines and growth factors are important regulators of FLS and chondrocytes.<sup>14–16</sup> Cytokines are categorized either as pro- or anti-inflammatory depending on their immediate effect on specific tissue, although there is considerable potential for pleiotropism depending on the cells targeted and the microenvironment. These regulators are ubiquitous in the synovium and synovial space and make their way either by filtration from plasma, or secretion by FLS, chondrocytes and the surrounding tissues.<sup>16</sup>

The joint is a dynamic environment that is subject to minor trauma continually, through movement, and in some joints, compression due to weight bearing, and is therefore subject to continued wound healing and repair processes. It is therefore necessary for articular cartilage and adjacent bone to be continually remodelled in a cycle of synthesis and degradation. This requires a balance of anabolic and catabolic enzyme activity in both cartilage and bone.

Carefully regulated proteolytic enzymes are responsible for the balance between anabolic and catabolic processes within the joint and cartilage.<sup>17</sup> Matrix degrading enzymes such as MMPs are present in normal synovial fluid, but are higher in concentration in RA, psoriatic arthritis (PsA) and osteoarthritis (OA).<sup>17–19</sup> The collagenases (MMP-1, -3, -8, -13 and -18) are the most important of these enzymes as they are the only known enzymes that can directly cleave collagen at neutral pH,<sup>20</sup> but other MMPs continue this breakdown when the triple helix collagen structure has become unravelled.<sup>21</sup>

Serine and cysteine proteinases are required to activate pro-MMPs (to MMPs) after they are secreted. Furthermore, inhibitors of these proteinases (e.g. tissue inhibitors of metalloproteinases (TIMPs), and inhibitors of serine proteinases (SERPINs)) are also present

in the normal joint. The levels and activity of these enzymes can be monitored indirectly by measuring their degradation products in the synovial fluid.<sup>22</sup>

### 1.3 The Inflamed Joint

The inflamed synovium has been studied at several levels: macroscopic, microscopic and molecular. Synovium is the primary target of disturbed immunomodulatory pathways in RA. Rheumatoid synovial tissue appears macroscopically hyperplastic and hypervascular (Figure 1A-B), while microscopically there is intimal lining layer hyperplasia and accumulation of inflammatory cells including T and B lymphocytes, plasma cells, macrophages, neutrophils, mast cells, natural killer and dendritic cells in the synovial sublining (Figure 1C-D).<sup>23</sup> Like the target organ in other autoimmune diseases (e.g. Sjogren's syndrome, autoimmune thyroiditis) infiltrating T and B cells have been demonstrated to form aggregates with varying degrees of organisation, and the potential to produce disease specific ACPA.<sup>24,25</sup>

Angiogenesis accompanies this cell accumulation, but it occurs in an abnormal manner resulting in different patterns of blood vessels associated with particular types of inflammatory arthropathies.<sup>26</sup> Additionally, the new blood vessels appear to be in an immature state.<sup>27</sup> The new vessels allow for increased leucocyte migration and the synovial tissue transforms into an invading 'pannus', that may cause cartilage and bone destruction.<sup>28,29</sup> Despite increased vascular supply, profound hypoxia in inflamed synovial membrane *in vivo* has been demonstrated.<sup>30</sup>

Many of the pathological changes manifest in the inflamed synovial tissue are observable in the synovial fluid (SF), which has also been studied intensively. Inflammation alters the permeability of synovial tissue.<sup>31</sup> In relation to RA synovium, the permeability to large molecules is increased, but that of small molecules is decreased (e.g urea and glucose). This is due to a combination of increased permeability of the vessels, cellular infiltration and synovial hyperplasia. The total protein content in SF is higher in inflammation and synovial inflammation inhibits the ability of the synovium to selectively filter proteins from entering and leaving the joint space.

The molecular weight distribution of the lubrication macromolecule hyaluronic acid (HA) is also altered, with a shift towards lower molecular weight forms in RA.<sup>32</sup> There is increased loss of HA from the joint and the mean HA concentration is lower in OA and RA synovial fluid.<sup>33,34</sup>

Pathological SF samples have markedly raised cytokine concentrations.<sup>35</sup> The role of cytokines in initiating and perpetuating the synovial inflammatory response continues to be studied intensively and has already lead to the development of several useful therapeutic agents, and the identification of further potential targets.<sup>16</sup> Changes in the cellular infiltrate of RA synovial tissue were recognised from an early stage to be associated with the clinical course of disease

and were used to identify specific responses to conventional and biologic disease modifying anti-rheumatic therapy.<sup>36–38</sup>

# Synovial biopsy

## 2.2 Retrieving Synovial Tissue Samples

The utility of synovial biopsy has been demonstrated in increasing our understanding of the pathogenesis of RA, in identifying potential therapeutic targets and evaluating current and new treatments.<sup>36–58</sup> It has also been proposed that synovial biopsies may give insight into the mechanism of action of a given agent.<sup>59</sup>

Synovial tissue may be obtained by needle biopsy, arthroplasty surgery, arthroscopically, or using ultrasound to guide the biopsy needle or grasping forceps (Figure 2).<sup>59</sup> Arthroscopic biopsy allows direct visualisation of the synovium and the operator can select an area of synovium to biopsy. Ultrasound depicts synovial thickness in greyscale and synovial vascularity with Doppler to assist in selecting a suitable biopsy site. While blind biopsy has been validated, arthroscopic and ultrasound guided biopsy are favoured by the majority of investigators for proof of concept experiments, as sampling is more specific for synovial tissue rather than connective tissue.<sup>53</sup>

Arthroscopic and ultrasound guided biopsy are safe and well tolerated. Data from 15,682 arthroscopies performed by rheumatologists revealed a complication rate for haemarthrosis of 0.9%, for deep vein thrombosis of 0.2%, and wound and joint infection both of 0.1%.<sup>60</sup> This incidence is reproducible at other centres where the overall complication rate was shown to be less than 0.3%. Similarly, an overall major complication rate is reported as 0.4% for ultrasound guided biopsy procedures.<sup>61</sup>

One study of biopsies in RA patients taken from both an inflamed knee joint and a small joint, showed similar mean cell numbers for all markers investigated in the synovial sublining in both sample sources.<sup>62</sup> Of further note, patients with clinically evident disease manifest at small joints have been shown to have similar abnormalities in clinically uninvolved knee joints, albeit at lower expression levels.<sup>62–64</sup> However, intimal lining layer hyperplasia appeared to depend on local processes ; there was no correlation of the numbers of intimal macrophages or FLS between the different joints. Consistent with these findings, there are differences in DNA methylation and transcriptome signatures in FLS from different joints of RA patients as well as differences in FLS invasiveness depending on their positional memory.<sup>65, 66</sup> Questions remain about where to biopsy within a given joint. In particular there have been concerns that mediators of inflammation may be differentially expressed in different parts of the same joint, especially between the cartilage pannus junction (CPJ) and non-CPJ sites which are known to behave differently. However for T-cells<sup>67,68</sup>, plasma cells<sup>68</sup>, several MMPs<sup>68</sup> and granzymes<sup>68</sup>, there have been similar results for biopsies from CPJ and non-CPJ sources. One study did find a difference for

macrophages<sup>63</sup>, but others did not.<sup>67,68</sup> Studies examining the number of synovial tissue specimens for reproducible research studies suggest at least six biopsy specimens is the optimal number.<sup>59, 70</sup>

Although synovial tissue analysis plays a minor clinical role in the differential diagnosis of arthritis (e.g. infectious, granulomatous, infiltrative diseases or crystal arthropathies), there are still profound unmet needs regarding predictors for diagnosis, disease progression and response to treatment. Therefore, studies of synovium have recently expanded beyond immunohistochemistry to involve methods of tissue digestion, homogenisation and indeed, whole tissue culture. The methods of examining synovial tissue at a molecular level include detailed 'omic' technology discussed in greater detail below. To this end the synovial tissue obtained from the joint is placed on saline-dampened gauze, snap-frozen in OCT or placed directly into RNAlater. The synovial fluid is centrifuged and a cell pellet may be isolated or separated, using Ficoll gradient, will provide synovial fluid mononuclear cells. Synovial fluid may reflect the synovial compartment better than blood but still provides only indirect information therefore studies of the synovium, the target tissue of RA is essential.<sup>71</sup>

Various prognostic biomarkers for RA have been identified in SF and validated in serum.<sup>72</sup> Studies performed using this strategy first identified proteins that may be of interest in the synovial fluid, and then searched for antibodies to these proteins in the plasma.<sup>73</sup> It is possible that this methodology may be useful in future experiments of synovial tissue, and the results of such research may be more easily translated into clinical practice.

Since 2002, cohorts of patients with early arthritis have been gathered. Having a cohort of early arthritis patients with clinical data, histological data, DNA and mRNA arrays plus proteomics is an instrumental resource for investigating differences in synovial tissue, comparing several inflammatory joint diseases with persistent self-limiting, persistent active disease as well as erosive and non-erosive disease.<sup>74</sup>

Most research with synovial biopsies has been performed from RA patients but some results suggest that synovial tissue sampling may be used in other inflammatory arthropathies such as psoriatic arthritis.<sup>71, 75-77</sup> This research aims to gain a fuller understanding of disease pathogenesis, mechanisms of action of current treatments, and the identification of novel targets and biomarkers. Indeed, the effects of various treatments for RA on the synovium has been studied, and the principal results of these data are presented in table 1.

## **2.2 Pathogenesis of Synovitis**

Synovial tissue inflammation is preceded by activation and proliferation of endothelial cells that form new blood vessels (angiogenesis) as one of the earliest changes. The newly activated vessels are the gateway for leukocyte infiltration into the synovium, actively recruiting immune cells into the tissue. This results in an expanding synovial pannus



characterised, in RA, by hyperplasia of the intimal lining layer and often invasion of pannus cells into the nearby bone producing erosions. Therefore, the synovium is the target tissue in RA and if studies are confined to examining cellular and molecular alterations in the peripheral blood then important information may be completely ignored. For example, Th17 cells are expanded in the blood of some RA patients, this lead to clinical trials of anti-IL17 monoclonal antibodies, however as limited Th17 expansion occurs within the synovium, this therapeutic approach achieved little effect<sup>78</sup>. Indeed, studies have shown enrichment of ex-TH17 cells at the site of inflammation in SF and ST from patients with RA<sup>79</sup>. Additionally, the primary invasive cells in RA are the FLS but these have not yet been associated with a circulating biomarker<sup>80</sup>.

## 2.3 Early Arthritis and Early Diagnosis

Some progress has been made in earlier diagnosis of RA , however signs of joint destruction may already be present at the time of diagnosis.<sup>81</sup> We know today that early, aggressive treatment is more successful than delayed treatment.<sup>82, 83</sup> Therefore the utility of biomarkers in securing a diagnosis as early as possible will allow treatment in the most timely manner, securing the best outcomes.<sup>84</sup> Those with undifferentiated arthritis may benefit most from this. Although ACPA are reasonably specific (96%), the diagnostic sensitivity in early arthritis is 57%.<sup>82</sup> Up to 30% of RA patients never develop ACPA.<sup>85</sup> An association has been defined between the presence of circulating ACPA and subsequent development of RA in subjects with arthralgia,<sup>87</sup> and with bone erosions in early untreated subjects.<sup>88</sup> Delay in diagnosis of RA may arise from either a lack of a definitive biomarker, or a failure to meet current diagnostic criteria, and these criteria have a significant reliance on biomarkers. Therefore, despite the advent of ACPA, there remains a need to identify further and specific susceptibility biomarkers. Recently, biomarkers for RA in those without detectable circulating ACPA have been identified, and this RA subset represent an important group to study, and may contribute greatly to our understanding of the disease pathogenesis.<sup>89, 90</sup>

The so-called 'at-risk of arthritis' cohorts have been the subject of much research in recent years. One potential corollary of this is the promise of cure and prevention of RA, where the initial break in self-tolerance is identified and targeted therapeutically.<sup>91</sup>

A positive ACPA status in those with arthralgia is associated with the subsequent development of arthritis in only about 20-30% of these people after 30 months of follow up.<sup>92, 93</sup> The synovial tissue of patients who are at risk of arthritis has also been examined in two relatively small studies Little evidence of synovitis was found in the first and subtle T-cell infiltration was noted in the second.<sup>94</sup> Data on alternative tissues such as lung and lymph node that may be important in the very early stages of arthritis as sites of first antigen presentation.<sup>94, 95</sup>

It is suggested that a 'window of opportunity' may exist during which RA can be most successfully treated. In initial studies few molecular differences had been observed in the

synovium comparing early and late disease.<sup>23, 96</sup> Recently, a highly expanded, specific T-cell clone has been identified in early RA, which underlines the importance of T-cells in early stage disease.<sup>97</sup> Epigenetic changes in FLS over time may define the different stages of RA after clinical onset of the disease.

In recent preliminary reports of ultrasound guided biopsies in unselected treatment-naïve, early arthritis patients compared to established RA, there was increased tissue expression of macrophage derived chemokines - CXCL4 and CXCL7 in RA only during the first 3 month window of symptom duration, and not later in disease.<sup>98</sup>

Synovial biopsy may be useful, as a diagnostic and prognostic tool, for the differential diagnosis in early inflammatory arthritis in whom synovial CD22 and CD38 expression may distinguish between RA and non-RA disease.<sup>99</sup> The value of synovial biopsy markers as selected diagnostic and prognostic markers to establish an early diagnosis is clearly still evolving.

A recent study included 50 patients with early arthritis, who had undergone synovial biopsy at inclusion and were followed for two years.<sup>100</sup> The focus was on the angiogenic processes in the initiation and perpetuation of synovial inflammation, in particular vascular endothelial growth factor (VEGF) and angiopoietins 1 and 2 (Ang-1 and Ang-2) and their tyrosine kinase receptors VEGFR and TIE-2. Expression of TIE-2 was significantly increased in the group with erosive disease as compared to the group with a self-limiting disease, and plasma-TIE-2 was significantly increased in the groups with persistent non-erosive disease and persistent erosive disease as compared to the group with self-limiting disease. Similarly, JNK activation is elevated in RA patients compared to undifferentiated arthritis, before classification criteria are met.<sup>101</sup>

Although more research is needed, these studies suggest that a synovial biopsy at disease presentation could be a useful tool for both patients and physicians, for early disease stratification into short self-limiting course versus severe persistent inflammatory course, and in the former between erosive versus non erosive disease, thereby informing the most appropriate treatment strategy.

## **2.4 'Personalised Medicine' and Disease Stratification**

In addition to diagnostic problems, predicting disease course is imprecise. Identification of a subgroup of early arthritis patients who will develop destructive disease may present a significant advance in selecting the most effective treatment for an individual patient.<sup>102, 103</sup> This is the so-called 'tailor-made' treatment, informed by biomarkers, used to assess what has been referred to as 'disease signatures'.<sup>98</sup> A more accurate description of this process is disease 'stratification'.<sup>104,105</sup> This concept proposes that a disease can be stratified into distinct

subsets that exhibit differential outcomes and responses, each subset labelled by a biomarker or combination thereof.

This is important as therapies are commonly selected on a trial and error basis, but less than 50% of RA patients experience a 50% improvement in their arthritis in response to any single biological therapy.<sup>106–108</sup> In the time that an ineffective treatment is administered, the disease may progress and patients may be potentially exposed to unnecessary adverse events. Therefore biomarkers that predict response to a given treatment will be of great clinical utility. Synovial biomarkers are likely to be of the greatest clinical utility and a great deal of work has concentrated on studying features of inflamed synovium in RA patients with samples taken after clinical improvement following treatment. More recently, a number of studies have analysed the predictive capabilities of synovial tissue biomarkers for disease course and response to therapy.

## **2.5 Recent advances**

Synovial tissue biopsy procedures and analysis are more widely available throughout the world.<sup>53</sup> This will inevitably enable a targeted approach to identifying biomarkers in synovial tissue.

With respect to disease stratification, sensitivity and specificity can be theoretically improved by combined use of biomarkers. For example a positive clinical response of RA to anti-tumour necrosis factor treatment with etanercept has been demonstrated using a biomarker signature generated by 13 autoantibodies and 11 cytokines. This study included three ethnically distinct populations, and for North Americans it demonstrated a positive predictive value of 71%, although independent validation is required.<sup>11</sup>

The advent of new proteomic, transcriptomic and genomic technologies, and the ability to combine clinical and radiological markers with these technologies, should make stratifying disease the norm in the future. It is possible that disease stratification will become so sub-categorised such that it is truly approaching personalised medicine. The ‘omics’ approach has been usefully applied to identify key players and protein interactions in several diseases. Studying the genome, the RNA or the protein will each have different sets of bias and variance, and it has been argued that combined approaches may lead to a more accurate assessment of important protagonists.<sup>104</sup>

Proteomics offers the advantage that the functional units of the cell are being studied directly, likely most accurately representing what is actually happening in the synovium. The development of technologies such as SomaLogics that have the power to measure thousands of proteins in small tissue volumes has the potential to allow a more complete characterisation of the disease network of RA. In RA the proteomics approach has so far focussed on peripheral blood mononuclear cells, serum and synovial fluid<sup>72,73,109</sup>; the

possibility that the synovial tissue itself may hold the key to unlocking the disease network has yet to be fully exploited. Furthermore, new technologies in protein separation, processing and identification are expected to increase proteome coverage.

In relation to transcriptomic analysis, microarray technology has been, to date, the most frequently employed strategy in the field of biomarker research. This facilitates the identification of candidate genes in pathophysiological processes. However, gene expression levels do not always predict protein levels, due to transcriptional and translational regulatory mechanisms and the activity of protein degradation processes.<sup>17</sup> A good example of the use of transcriptomic data was demonstrated while determining a rule-based classification that allows differentiation between RA and osteoarthritis.<sup>110</sup>

Microarrays contain probes for thousands of different genes that makes them suitable for screening large cohorts. The high throughput techniques used in transcriptomics, however, also allow detection of significant gene expression differences with modestly sized cohorts.<sup>111</sup> Transcriptomic analysis is already being used to examine the gene signature of synovial tissue, augmented by the newer sequencing technologies that permit deeper transcriptional coverage than microarrays, including spliced variants.

There have been a number of studies where DNA array technology used to study gene expression in RA has been shown to be a useful and practical methodology. Different subtypes of RA patient synovia have also been characterised by gene expression analysis.<sup>112,113</sup> Gene-expression variance among RA patients has been described impacting several pathways involved in cell proliferation, cell survival, angiogenesis and regulation of inflammation.<sup>114</sup>

## **Biomarkers**

### **3.1 General Considerations**

### **3.2 Cellular Composition**

Simple cell count densities (or cellular infiltration) were appreciated as synovial tissue biomarkers associated with RA, more than 20 years ago. In a study published in 1989, a group showed that there was a decrease in T cell numbers after at least 6 months of gold treatment. They also reported a reduction in the ratio of T-helper cells to T-suppressor/cytotoxic cells in those who were treated successfully. Macrophages were not reported in this study. Furthermore, the number of biopsy samples where B cells could be identified decreased from 36% before successful treatment to 7% after treatment.<sup>36</sup>

The most convincing evidence for a cellular biomarker of treatment response points to the use of CD68 macrophages. Patients taking prednisolone had a reduction in synovial macrophages, expressing cell surface CD68, after two weeks of treatment compared with controls. The reduction in CD68 staining cells was mostly attributable to a decrease in the number of macrophages localising to the synovial sublining. There was also a decrease in the CD4 and CD5 (T and B cells) and CD38 (plasma cells) and CD55 (fibroblast-like synoviocyte (FLS)) cells.<sup>42</sup>

In an earlier study a reduction in synovial macrophages was observed 12 weeks after gold therapy. CD68 cells were most commonly identified in all layers independently of the site of synovial biopsy. No significant change in lymphocyte numbers was noted following treatment.<sup>115</sup>

A significant reduction in macrophages in the synovial sublining region following treatment with disease-modifying antirheumatic drugs (DMARDs), mostly methotrexate and gold, was demonstrated in another study.<sup>116</sup> This was particularly pronounced in those who were responding clinically by ACR criteria. Although a significant reduction in memory T cells was observed, (and this was interestingly associated with CRP reduction), memory T-cells could still be found in the synovium of patients who attained remission.

Synovial macrophages were also significantly reduced in the sublining after 16 weeks of treatment with leflunomide, and in the intimal lining layer after 16 weeks of methotrexate monotherapy. Synovial T cell numbers were decreased with both treatments but did not reach statistical significance in this study.<sup>41</sup> While in a similar study, after 16 weeks of methotrexate, a decrease in synovial CD3, CD8, CD38, CD68 and Ki67 were demonstrated to be statistically significant. Notably CD4 infiltration was not reduced in this study.<sup>117</sup>

Treatment with infliximab has been shown to reduce CD3 (T cell) in repeat synovial biopsies taken 4 weeks after treatment, and this finding was associated with clinical response.<sup>39</sup> In ten patients with longstanding RA who received infliximab, reduced numbers of synovial CD3 (T cells), CD22 (B cells) and CD68 macrophages, 2 weeks after treatment were demonstrated in a separate study.<sup>45</sup> In a study of infliximab versus placebo in which 24 patients with active RA underwent arthroscopy and biopsy before, and 48 hours after infliximab, revealed a significant reduction in CD68 intimal macrophages, as well as a non-statistically significant reduction in CD68 macrophages, T cells and plasma cells in the sublining. In a large prospective study in 143 RA patients the clinical response to infliximab was predicted by the number of TNF producing cells, CD68 macrophage subsets and synovial expression of TNF.<sup>118</sup> In a follow-up study, the number of lymphocyte aggregates was also predictive of the clinical response.<sup>119</sup> Positivity for lymphocyte aggregates increased the power to predict the clinical response, when analyzed in a prediction model that included baseline disease activity evaluated by the Disease Activity Score in 28 joints, ACPA positivity, and synovial TNF expression.

Anakinra, a specific IL-1 antagonist, has also been shown to significantly reduce the synovial intimal macrophage population.<sup>43</sup> A significant decrease of intimal lining CD68 macrophages was observed following rituximab infusion in responders.<sup>54</sup>

Synovial response to rituximab has also been assessed by serial biopsy. A series of 6 patients with seropositive RA who had synovial biopsy before treatment with rituximab, and then 4 were re-biopsied after 8 weeks has been reported. The authors assert that although it is known that rituximab depletes circulating B cells, as well as B cells in salivary glands, little is known of its effect on synovial tissue. 4/6 agreed to follow up biopsy. 2 had complete depletion of CD20, 1 had no change and 1 biopsy was insufficient to analyse.<sup>120</sup>

In other studies rituximab induced a similar variable depletion in synovial B cells with an indirect decrease in macrophages, T cells and plasma cells at the time of the clinical response.<sup>121,122</sup> Of interest, the change in plasma cells was associated with the clinical response.

The utility of CD68+ macrophages in the sublining layer as a candidate biomarker was systematically tested across different interventions and kinetics.<sup>123</sup> It was shown that changes in numbers of synovial sublining CD68+ macrophages correlate with clinical improvement independently of the therapeutic strategy. A study designed to determine if the correlation between the change in number of synovial sublining CD68 cells and change in DAS28 was confirmed in a multicentre study with excellent inter-centre agreement.<sup>124</sup> The number of CD68 macrophages decreases with a reduction in disease activity as measured by the DAS, thus demonstrating that CD68 numbers could be used as a biomarker of therapeutic response.<sup>123</sup>

Furthermore, synovial CD68 expression is superior to clinical evaluation as it is less susceptible to both the placebo effect and expectation bias (59% of delegates at OMERACT agreed)<sup>123, 125</sup>. It could therefore be used to assess the therapeutic efficacy of novel treatments.<sup>124</sup> In conclusion, synovial macrophage CD68 expression as a synovial biomarker demonstrates validity, reliability and feasibility as a biomarker of disease activity and response to treatment both in early and established RA.

A number of studies addressed whether markers of synovitis are associated with clinical phenotype or development of a persistent, erosive disease course. In two large studies in established RA, large lymphocyte aggregates were found in around 30% of patients but did not associate with a clinical phenotype.<sup>126,127</sup> In early arthritis, the presence of lymphocyte aggregates did not predict an aggressive disease course and aggregates were rapidly diminished by several anti-rheumatic treatments.<sup>119,128</sup> Taken together, these studies suggest that lymphocyte aggregates are a pro-inflammatory phenomenon and not a persistent primary driver of synovitis. In contrast, as described above, biomarkers of angiogenesis, namely the activation of the tyrosine kinase receptor TIE-2, as well as increased JNK expression predicted an aggressive disease course in early arthritis patients.<sup>101</sup>

### 3.3 Cytokines

The increased expression of several cytokines in the inflamed synovial tissue is well established, and for TNF and IL-6 concentrations correlation with disease activity, independent of disease duration, has been demonstrated.<sup>27</sup> The levels of CCL2/MCP-1 were also found to be increased in RA serum and synovial tissue.<sup>50</sup>

With regards to treatment effect, the expression of IL-1 $\beta$  and TNF was 40% (95% CI 18–56%) and 52% (95% CI 10–74%) respectively lower following prednisolone therapy compared with placebo. Notably, this effect was mainly attributable to changes in the synovial sublining, and appeared to correlate with clinical improvement.<sup>42</sup> Significant cytokine expression changes after 12 weeks of gold treatment, in three areas of synovium; lining, perivascular and connective tissue, have also been reported. In the intimal lining layer, IL-1 $\alpha$ , IL-1 $\beta$  and IL-6 levels were statistically significantly reduced after treatment, and this seemed to correlate with clinical response. TNF was also reduced in all three areas, but did not reach statistical significance in the lining.

TNF was only slightly reduced in synovial samples after 16 weeks treatment with either methotrexate or leflunomide. IL-1 $\beta$  was only moderately reduced in the leflunomide-treated patients while reductions in the methotrexate patients were significant, which highlights potential different mechanism of action between DMARDs.<sup>41</sup> In a separate study IL-1 $\beta$ , but not IL-1 $\alpha$  was shown to have a statistically significant reduction in expression after 16 weeks of treatment with methotrexate, and this again seemed to correlate with clinical response.<sup>117</sup>

In biopsy samples of ten active RA patients taken 2 weeks after infliximab, IL-8 and MCP-1 were shown to be reduced in both the lining and sublining, and, despite a downward trend in synovial expression of Gro  $\alpha$ , RANTES, and MIP-1b this was not significant.<sup>45</sup>

Acute serum amyloid A (A-SAA) expression and production has been demonstrated in RA synovial tissue and A-SAA induces angiogenesis, cell matrix interactions, chemokine and MMP expression in RA.<sup>130</sup> A-SAA has a significant role in the inflamed joint increasing expression of MMP-1, MMP-3, MMP-13, and MMP/TIMP expression in RA FLS and synovial explants. Furthermore, blockade of its receptor (scavenger receptor class B type 1 (SR-B1)) and TLR2 inhibited migration and invasive mechanisms. Importantly, A-SAA has the ability to induce TNF expression in RA synovial explant cultures, while baseline serum A-SAA levels correlated with the 28-joint swollen joint count and 1-year radiographic progression independently. Therefore, A-SAA is a promising biomarker of disease activity both in the synovium and in the serum.<sup>131</sup>

### 3.4 Chemokines

Leucocytes are attracted to the target tissue by soluble chemotactic cytokines termed chemokines, released from activated cells in the tissue to stimulate leucocyte migration through the endothelial barrier.<sup>132</sup> The chemokines IL8/CXCL8 and MCP-1/CCL2, among others, are expressed abundantly in RA synovial tissue. Previous work has shown that the development of clinical signs of RA synovial inflammation is specifically associated with increased synthesis of the CXCL8<sup>133</sup> and expression in synovial tissue reflects response to therapy in RA patients. In addition, a proof of concept study of an oral CCR1 antagonist in RA patients showed significant reduction in synovial macrophages and chemokine expression.<sup>134</sup>

### **3.5 Growth Factors/Adhesion Molecules**

ICAM-1 expression was significantly reduced in patients treated with both leflunomide and methotrexate. Notably, a decrease in ICAM-1 was seen in those that responded to leflunomide and to methotrexate, while non-responders did not experience a statistically significant decrease. VCAM-1 was reduced in both groups, but this difference was significant only in the leflunomide-treated patients.<sup>41</sup>

Another study demonstrated that VCAM-1 and E-selectin were both statistically significantly reduced in expression after 16 weeks of treatment with methotrexate, but in this study ICAM-1 did not reach statistical significance.<sup>117</sup> Treatment with infliximab has been shown to reduce VCAM-1 and E-selectin in repeat biopsies taken 4 weeks after treatment.<sup>39</sup>

In relation to treatment with anakinra, a patient taking the dose of 150mg/day, was shown to have a 74% reduction in synovial membrane E-selectin, but the 5 patients taking the lower dose (30mg/day) did not demonstrate this. No change was seen in P-selectin. A significant decrease in ICAM-1 and VCAM-1 was seen in the high dose patients, and a small decrease in 2 of the 5 on the lower dose of anakinra.<sup>43</sup>

### **3.5 Mediators and Products of Bone, Cartilage and Tissue Degradation**

Type II collagen is the main collagen of articular cartilage, and is excessively degraded in RA. It is known that collagen biomarker and MMP levels predict radiographic progression of RA, and therefore may act as a prognostic biomarker.<sup>135, 136</sup> A study with the primary objective of attempting to understand more about the mechanism of action of methotrexate (19 subjects) and leflunomide (16 subjects) demonstrated that MMP-1 was significantly reduced by both. The level of TIMP-1 was significantly reduced in the leflunomide-treated patients, but not the methotrexate-treated patients. Furthermore, both drugs reduced the overall expression of MMP-1 and the MMP-1: TIMP-1 ratio after 4 months of treatment. The changes were more pronounced in patients who fulfilled the ACR 20% response criteria.<sup>41</sup>



A number of studies analyzed the effect of immunomodulatory treatment on synovial mediators of bone destruction. Treatment with both infliximab and etanercept increased the expression of osteoprotegerin (OPG) in synovial tissue and had no effect on RANKL, resulting in an increased OPG:RANKL ratio.<sup>49</sup> In contrast, rituximab induced a 99% decrease in receptor activator of nuclear factor  $\kappa$ B (RANK)-positive osteoclast precursors and a decrease of 37% in RANKL and a trend towards reduced synovial OPG expression. In serum, both OPG and RANKL levels were significantly reduced, but the OPG/RANKL ratio increased (157%).<sup>137</sup> Finally, abatacept did not exert a significant effect on synovial OPG, RANK or RANKL mRNA expression in a study in 16 patients.<sup>138</sup>

The family of S100 proteins are a closely related group of low-molecular weight (9–14 kDa) acidic calcium-binding proteins. Originally described in oesophageal epithelium, as well as neutrophils and macrophages, they are involved in calcium dependent cell activities such as cytoskeleton regulation and cell migration and adhesion. The extracellular role of these proteins is of interest as they have been found to be overexpressed in inflammatory compartments. They are in effect pro-inflammatory cytokines. S100A12 has important activities in relation to innate and acquired immune responses.<sup>139</sup> One study using quantitative proteomics demonstrated an association between the severity of joint erosion in RA and the S100 proteins A8, A9 and A12 levels.<sup>109</sup> The S100 proteins myeloid-related protein (MRP)-8 and MRP-14 regulate myeloid cell function and control inflammation. Before initiation of treatment, responders to targeted treatments showed significantly higher MRP8/14 protein complex levels compared with non-responders. Moreover, in responders to adalimumab, infliximab or rituximab treatment, MRP8/14 levels decreased after 4 weeks of treatment, but not non-responders.<sup>140</sup>

### **3.6 Antigens and Antibodies**

Expression of antigenic proteins has been described in RA synovial tissue. The presence of deiminated proteins, such as the  $\alpha$ - and  $\beta$ -chains of fibrin, in RA synovium appeared to be major antigenic targets of ACPAs.<sup>141</sup> In addition, anti-Sa antibodies that recognize deiminated vimentin appear to be specific for RA and have been isolated from RA synovium.<sup>142</sup> Finally, intracellular citrullinated proteins colocalizing with ACPA reactivity have been demonstrated in RA synovium.<sup>143</sup> but the presence of citrullinated antigens is not specific for RA synovial tissue.<sup>144</sup> It has also been shown that anti-filaggrin antibodies are produced by local plasma cells resident in the RA pannus.<sup>145</sup>

### **3.7 Genes and Transcripts**

In a study in 18 RA patients treated with infliximab several biological processes, related to inflammation, were up-regulated in pre-treatment synovial tissue biopsies in patients who

responded to therapy.<sup>146</sup> In a larger follow-up study, Lindberg *et al* reported the results of RNA analysis of synovial biopsies of 62 patients with RA before treatment with infliximab. They found that the presence of lymphocyte aggregates dominated the expression profiles and that there was a significant overrepresentation of lymphocyte aggregates in patients who had a good response, which confounded the analysis. Nonetheless, in those that were lymphocyte aggregate positive, 38 transcripts were associated with differences between good and non-responders.<sup>147</sup>

In a study of paired synovial biopsies of RA patients, before and 12 weeks after adalimumab, genes were differentially expressed between biopsies of responders and non-responders. These genes could be split into two distinct families: genes involved in the regulation of immune responses and genes involved in the regulation of cell division. To confirm the microarray findings, synovial expression of selected molecules was assessed using specific antibodies. Synovial expression of IL-7R, CXCL11, IL-18, IL-18rap, and MKI67 was significantly higher in poor as compared with moderate and good responders, thereby serving as a potential biomarker of response to adalimumab.<sup>148</sup>

In another study of paired synovial biopsies of RA patients before and after rituximab treatment clinical responders demonstrated higher expression of macrophage and T cell genes, while clinical poor responders showed higher expression of interferon- $\alpha$  and remodelling genes.<sup>149</sup>

### 3.8 Other

The properties of the cells in the inflamed synovium differ markedly from normal cells. Profound hypoxia in the inflamed synovial membrane has been described *in vivo*.<sup>30</sup> Low tissue partial oxygen pressure (tPO<sub>2</sub>) levels in the inflamed synovial joint tissue are significantly associated with increased markers of macroscopic and microscopic inflammation. There is an association of tPO<sub>2</sub> with macroscopic synovitis as well as CD68 and CD3 cell infiltrate in the sublining, and various pro-inflammatory cytokines (TNF $\alpha$ , IL1 $\beta$ , IFN $\gamma$  and the chemokine MIP3 $\alpha$ ). When primary synovial fluid cells were exposed *in vitro* to pO<sub>2</sub> levels, similar to those in the inflamed joint, there was a significant increase in cell migration.<sup>150</sup>

## Conclusion and Current Limitations

Much of the work on serial synovial biopsies has been performed on patients with known diagnoses, and has been performed to investigate responses to treatment. There remains a critical need for identifying biomarkers for diagnosis which can be applied in clinical practice.

Biomarkers may reduce the time taken and the number of patients required to screen for the potential efficacy of new drugs.<sup>52,151</sup> The number of patients with active disease eligible to participate in studies is limited. As with all trials, the number of patients who are to be put at risk by exposure to drugs at an early stage of development, as well as to be placed on placebo, are restricted by ethical considerations.<sup>152</sup>

Although finding biomarkers in peripheral blood is attractive because it is more feasible and less invasive than synovial biopsy, since inflamed synovium is the ultimate target of inflammation, it should be a potentially rich source of potential biomarkers. Furthermore, many confounding factors might interfere with peripheral blood profiles. Some authorities have suggested that a more targeted approach to searching for serum markers should be to first identify potential biomarkers in the inflamed synovial joint, and later studying the plasma for the presence of the same biomarker.<sup>140</sup> Such an approach in RA patients has demonstrated clinical utility when candidate peripheral biomarkers of synovial pathotype predicted response to biologic therapy.<sup>153</sup>

Although new technology has enabled faster and more complete analyses of proteins, because of the high complexity in protein and protein isoforms in the synovial joint, interpreting the results of 'shotgun' proteomics is a challenging endeavour.

New technologies are experiencing difficulties. Expression levels from three widely used microarray platforms demonstrated poor reproducibility.<sup>154</sup> In addition, as there are high levels of background signals in array datasets, there is a decreased sensitivity to transcripts present in low numbers.<sup>155</sup>

The development of high quality immunoanalytical assays can be slow and expensive. This makes the verification of candidate biomarkers a challenging process, and at the moment, despite the increase in availability of means to biopsy synovial tissue, there remains a lack of diagnostic makers.

While there are a great many genomic biomarkers that can predict response to treatment, or those at most risk of adverse events in many areas of medicine, rheumatology appears to have experienced only limited benefit from this emerging field. Many studies have attempted to identify biomarkers to predict response to anti-TNF treatment, but to our knowledge, only those reported above have used synovial tissue to search for these.<sup>119,147,148</sup>

In addition, where data from control synovial tissue specimens is available, osteoarthritis is often used as a disease control, although it is increasingly recognised that osteoarthritis has

an underlying inflammatory response, albeit significantly limited and less associated with specific autoimmunity than RA.

It is likely that in the near future a reasonable goal will be to stratify disease before the phenotype is established, and this represents an early step toward eventual ‘personalised medicine’. Identifying surrogate markers is therefore an aim for which synovial tissue is an indispensable research tool.

	Cells	Molecular marker	Implications	References
Pathogenesis of disease	Fibroblast-like synoviocytes	DNA methylation	Jak-STAT pathway <i>HOX</i> genes	Ai, R., et al. Nat Commun. 10, 1
	Macrophages	CD68 surface antigen	Central effector cell	Tak PP, Bresnihan B. Arthritis Rhe
	T cells	CD3 / CD45Ro / Th17	Pivotal immune cells	Kobezda, T., et al. Nat Rev Rheu
	B cells	CD20 / CD22	Production of antibodies; T-cell cooperation	Humby, F., et al. PLoS Med 6, e
Changes upon targeted therapy	Macrophage	CD68	Correlates with change in disease activity	Dolhain, R. J., et al. Rheumatol
	T cells	CCR7		Smith, M. D., et al. Rheumatol 4
	B cells	CD20 / CD22		Walsh, C. A. E., et al. Clin. Exp. F
Candidate synovial tissue biomarkers including proteins and genes		ASAA MRP8/14 ICAM-1 MMP-1/-3 CCR1 OPG/RANKL TIE-2 JNK	Many are measurable in the synovium and in the circulation	Mullan, R.H., et al. Arthritis Rhe Choi, I.Y., et al. Ann Rheum Dis Kraan, M. C., et al. Arthritis Rhe Catrina, A.I., et al. Arthritis Rhe Haringman, J.J., et al. Ann Rheu Catrina, A.I., et al. Rheumatol 4 Boumans, M.J., et al. Ann Rheu de Launay, D., et al. Ann Rheum
Synovial markers of remission		CD68		Smith, M. D., et al. Rheumatol 4

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